

## **Redox proteomics applied to the thiol secretome**

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**Abstract**

Studies of redox proteomics to identify glutathionylated proteins have so far been focused on intracellular proteins. However, plasma proteins have been described as glutathionylated. The present review discusses the redox state of protein cysteines in relation to their cellular distribution. We focus in particular on the redox state of proteins secreted under inflammatory conditions and that may act as soluble mediators (cytokines). We describe the various approaches used to detect secreted glutathionylated proteins, the only thiol modification studied so far in secreted proteins, and the specific problems presented in the study of the secretome. This review will deal with a particular aspect of redox proteomics, the analysis of secreted proteins and their possible function.

## **Redox state of proteins in the context of their localization**

### *Redox proteomics of the thiol proteome*

According to the Oxford English Dictionary, proteomics is “the study or analysis of the set of proteins expressed by an organism” and we commonly apply the term to high-throughput techniques of protein identification. As such, the history of redox proteomics started in 2002 when various groups published the first reports of the identification of oxidized proteins by modifying the existing proteomic techniques. Two of these papers were aimed at carbonylated proteins (19,20) and others at protein glutathionylation (33,37,61) or overall protein thiol oxidation (60) .

This review will deal with the redox states of protein thiols. Biochemistry textbooks and protein databases classify cysteines in proteins as either free or engaged in a disulfide bond. However, “free” cysteines can be reversibly or irreversibly oxidized to various forms. In particular, in addition to forming structural disulfides, cysteines can form transient, reversible disulfides with another protein cysteine, with free cysteine (cysteinylation) or with the cysteine in the tripeptide glutathione (GSH; glutamyl-cystenyl-glycine). Fig. 1 lists the most important oxidation state of sulfur in cysteine. These forms of oxidation, due to their reversibility, are of great interest in the context of redox regulation, as a means by which the redox state of the cell can regulate protein functions (43-45).

Specific proteins undergoing glutathionylation were described even before the term “proteomics” became popular (in the late 1990s, according to Google books ngram viewer - <https://books.google.com/ngrams>). Two proteins have been identified as undergoing glutathionylation in two separate papers, an unidentified 30 kDa cytosolic

protein in oxidant-treated rat liver (79), and actin in human neutrophils during oxidative burst (22). Although these were not the result of high-throughput studies, some of the technologies used were then adapted for two-dimensional gels for further studies and are at the basis of current redox proteomic techniques. In particular, the first published paper using the expression “redox proteomics” (37) was substantially based on the application of the technique described by Rokutan et al. (79) for 2-dimensional (2D) gels.

Most redox proteomic studies published to date report the identification of cytosolic proteins because the analyzed samples were cell lysates. Studies by the group of Mike Murphy, specifically focusing on mitochondria, has identified several mitochondrial proteins susceptible to thiol oxidation (60). Protein abundance is also a key factor in the identification of oxidized proteins. For instance, when we tried to identify specific biochemical pathways targeted by protein glutathionylation, we could identify glycolytic enzymes, as shown in Fig. 2, suggesting that glycolysis is potentially regulated by the redox state of protein thiols. In fact, Fig. 2 also shows that many of the glycolytic enzymes undergoing glutathionylation were known in the literature for their enzymatic activity to be affected in by thiol oxidation. This would predict that a shift in the GSH/oxidized GSH (GSSG) ratio may affect the overall anaerobic energy metabolism. However, the focus on glycolytic enzymes may be due to the fact that they are abundant proteins, which preferentially identified by most proteomics techniques (75).

### *Subcellular localization of the thiol redox state*

This section will discuss the redox state of protein cysteines in different cellular compartments. It will provide some reason for the high prevalence of cytosolic proteins among those undergoing transient post-translational modifications (PTM), including glutathionylation, and possibly identify understudied areas worth exploring.

*Cytosol.* The intracellular environment is considered a highly reducing one, mainly due to the 1-10 mM concentrations of GSH, at a high GSH/GSSG ratio (up to 100/1). This is an unfavourable condition for the formation of disulfide bonds and, in fact, structural disulfide bonds are normally formed in the endoplasmic reticulum that is a more oxidizing environment (GSH/GSSG, 1/1) (50).

*Plasma.* On the contrary, the extracellular environment is oxidizing. The main free thiol in serum/plasma is represented by cysteine, but that is only in the range of 10  $\mu$ M and with a reduced/oxidized (cysteine/cystine) ratio of 1/6 (9). GSH is also present in plasma but in the range of 1  $\mu$ M (1,000-fold less than in the cell) and at a GSH/GSSG ratio around 1 (9). Other studies also reported that plasma GSH+GSSG is present at at least 10-fold lower concentrations than Cys+cystine but while cysteine is mainly oxidized (Cys/cystine ratio around 1/10), glutathione is more reduced, even extracellularly (GSH/GSSG ratio of 10/2) (57). The same study shows that significant amounts of cysteine and GSH are present in the oxidized form in plasma as mixed disulfides with proteins: 40% of total plasma cysteine is present in cysteinylated proteins and 70% of plasma GSH is oxidized as mixed disulfides to

form glutathionylated proteins (57); by comparison, Brigelius et al. (14,15) reported that, in normal liver, 1% of intracellular GSH is present as glutathionylated proteins. Hence, even if total plasma glutathione is 1/10 of total plasma cysteine/cystine and is predominantly in a reduced state, the plasma concentration of glutathionylated proteins is slightly higher than that of cysteinylated proteins, raising the interesting question of whether protein glutathionylation occurs before secretion. Fig. 3 summarizes the concentration and redox state of thiols in the cell and in plasma that, although coming from the studies cited above and obtained in different models, can provide an idea of the order of magnitude of the different redox states of cysteines.

#### *Structural disulfides in cytoplasmic versus secretory proteins*

Most studies on protein structure point out that cytosolic proteins have no disulfides while extracellular proteins have no or little free cysteines (34,36,90). Butera et al. (17) have calculated that, based on Uniprot annotations for 4,000 proteins, about half of the disulfide bonds are in membrane proteins and the other half in proteins containing a secretion signal sequence, but about 5% disulfide bonds are found in nucleoplasmic or cytoplasmic proteins.

Studies using diagonal electrophoresis in primary cardiac myocytes or a neuronal cell line have shown that cytosolic proteins can form disulfide bonds upon exposure to oxidants such as diamide or hydrogen peroxide (13,30). These studies introduce the class of transient disulfides, a concept that Linke and Jakob summarized with the title "Not every disulfide lasts forever" (62). Disulfides also vary in their surface exposure and while the most surface-exposed are easily reduced, those buried in the three-dimensional structure of the protein require prior denaturation of the protein

(16). Accessibility is also important for a cysteine to be susceptible to glutathionylation, because of the size of GSH (307 kDa). This is even more important when glutathionylation occurs by thiol-disulfide exchange with GSSG (612 kDa) (45), and even more if this reaction is catalyzed by glutaredoxin (63). It is likely that transient disulfides are also surface-exposed. These disulfides can be intraprotein or interprotein ones and Brennan et al. noted that many of them imply associations with other proteins (13).

#### *Protein thiols on the plasma membrane*

The plasma membrane is at the interface of the intracellular environment and the extracellular one. We might expect, therefore, that protein thiols on the inner side are prevalently reduced and the ones on the exofacial side prevalently oxidized. We are not aware of studies attempting a quantification of the redox state on protein thiols on the two sides of the membrane.

However, a few papers reporting the existence of cell surface, exofacial, thiols have received some attention. Early studies on lymphocytes showed that their surface thiols increased upon mitogenic stimulation (58). Studies overexpressing or inhibiting protein disulfide isomerase (PDI) showed that this enzyme is important for keeping exofacial thiols in the reduced state (53). Thus, an active mechanism could be involved in the presence of free thiols on the exofacial side of the plasma membrane despite the extracellular environment being oxidizing. Redox proteomics has been successfully applied to identify membrane protein whose exofacial thiols are redox-sensitive (55,56).

## **The secretome**

With the term secretome we refer to proteins that are released in the cell culture supernatant or found in circulation. We are aware that a conservative use of the term "secretion" would restrict its use to proteins that are secreted through the classical pathway and have a signal sequence for targeting to secretion via the Golgi system. However, the term "non-classical secretion" is often used to define non-classical mechanisms; hence our use of the term "secretion" instead of "release" in this review.

### *Oxidized proteins in circulation*

To our knowledge, only a few plasma proteins have been studied in terms of their redox state.

*Serum albumin* is a major plasma protein, probably the most abundant secretory protein, and is present in serum at concentrations of 30-50 mg/ml. Human serum albumin has 17 cysteines of which only one (Cys34) is not engaged in a structural disulfide. Due to the low concentrations of free thiols in plasma, albumin may well be the main thiol antioxidant in circulation (76,94). This can undergo oxidation to form a mixed disulfide with cysteine (cysteinylation), homocysteine (32) or a sulfinic acid (66). Increased levels of cysteinylated albumin have been associated with intrauterine growth restriction (3).

*Transthyretin* is a transport protein (the name originates from "transports thyroxine and retinol"), also involved in amyloidosis (83). It can be found in circulation in the 0.5 mg/ml range as S-cysteinylated, S-cysteinglycinylated and glutathionylated (42,84).



*High-mobility group protein box 1 (HMGB1)* is a nuclear protein that can be released and act as a pro-inflammatory danger signal (97). HMGB1 has 3 redox-active cysteines (Cys23, Cys45, Cys106). The different redox state of these cysteines also regulates the pro-inflammatory activity of HMGB1 (103). In circulation, it can be found with an intramolecular disulfide between Cys23 and Cys45 or sulfonlated on all three cysteines and its oxidation is affected in acetaminophen poisoning (103).

*Pentraxin 3 (PTX3)* is an inflammatory protein related to C-reactive protein that is elevated in a number of infectious, inflammatory and cardiovascular diseases (12). Formation of disulfide-linked oligomers of PTX3 has been reported and one study has shown that measuring its oxidation state can provide a superior biomarker for sepsis than measurement of total PTX3 (29).

### **Studies specifically addressing the proteomic identification of the redox secretome**

Despite redox proteomics has been applied to the thiol proteome looking at different forms of thiol oxidation (S-nitrosylation, transient disulfides, mixed disulfides), so far high-throughput proteomics of the thiol secretome has only been applied to protein glutathionylation. However, these are very recent studies and hopefully they will be followed up by investigations on other types of thiol oxidation. In particular, given the high concentration of extracellular cysteine it will be important to identify the proteins released as cysteinylated.

We have specifically asked if proteins can be released as glutathionylated by inflammatory macrophages. To answer the question, we have used a strategy

employing biotinoylated GSH ethyl ester (BioGEE), originally used to identify glutathionylated proteins in HeLa cells (88). The principle is based on labelling the intracellular GSH pool with biotinylated GSH. However, because GSH is poorly cell permeable, its ethyl ester is used (98). This reagent is now commercially available and has been used in over 150 papers on protein glutathionylation.

The experimental design is based on previous publications from other groups. Basically, cells are preincubated for 2 hrs with BioGEE, washed and then cultured in reduced-serum medium (Optimem; not completely serum free and not defined as it contains a proprietary mixture of growth factors) with or without an inflammatory stimulus (lipopolysaccharide, LPS). After harvesting the supernatant, free thiols are blocked with an alkylating agent (such as N-ethyl-maleimide, NEM) to prevent thiol disulfide exchanges. Then proteins are bound on streptavidin-agarose and analysed by mass spectrometry. The proteins on the beads can be directly digested with trypsin for analysis of tryptic peptides or eluted with dithiothreitol (DTT). The latter approach should reduce the chances of identifying proteins non-specifically binding to streptavidin or to agarose and enrich those that are attached to streptavidin through a disulfide bond (Fig.4).

Once a list of proteins is generated by the mass spectrometry (MS) analysis, one has to bear in mind that this proteomics strategy is not quantitative but is highly sensitive and has a number of bias; for instance, the proteins identified could still be bound non-specifically to the beads, or be a result of the non-specific leakage from a small number of dead cells. Having identified a protein in a LPS-stimulated supernatant does not imply that its secretion is induced by LPS, also because there will always be a small amount of secretion by resting cells, or, again, due to a small amount of dead cells. Therefore, it is imperative that each protein is validated. In our

studies, we normally provide the list of the identified proteins as supplementary data but report only those validated in the main body of the manuscript (23). This makes it possible for others to eventually validate other proteins of interest.

In fact, in order to claim that a protein in the list of those identified by MS is released as glutathionylated and its release is induced by LPS, both arms of the claim need to be confirmed. Typically, a Western blot is required to measure the protein in the supernatant of LPS-stimulated versus unstimulated cells. This should be done with a proper number of replicates and, possibly, followed by densitometry and statistical analysis (23). Of course, if an enzyme-linked immunosorbent assay (ELISA) or any other assay (such as an enzymatic assay in case the protein is an enzyme) are available, that would be preferable as it may be more quantitative than a Western blot. Demonstrating that a protein is secreted as glutathionylated is more difficult as it requires immunoprecipitation with an antibody against the specific protein followed by non-reducing gel electrophoresis and Western blot with an anti-GSH antibody. Although we have used this approach, we feel it is not always satisfactory due to the presence of non-specific bands and the non-optimal migration in non-reducing conditions that does not allow an exact estimate of the molecular weight of the various bands. A control where the sample is run under reducing conditions, to remove GSH from the protein, will help. Finally, checking on a database, such as Uniprot, that the “glutathionylated” proteins identified actually have a free cysteine available for glutathionylation will avoid embarrassing mistakes.

In some cases, of course, the glutathionylated protein may have already been detected by the MS analysis of the proteins or their peptides at the stage of the identification. Identification of a glutathionylated peptide will also provide the additional information of which cysteine is glutathionylated. However, many protocols

for MS analysis include a reducing step, making thus impossible to identify glutathionylated peptides.

## **Proteins in the redox secretome**

### *Glutathionylated proteins*

Table 1 lists proteins secreted as glutathionylated by LPS-stimulated macrophages identified as described above. Below we discuss some of them.

*Redoxins* Three proteins in the list are involved in redox regulation and were the first ones we validated. In fact, we had previously identified by redox proteomics intracellular peroxiredoxin (PRDX) 1 (37) and thioredoxin (TRX) 1 (18) among the proteins undergoing glutathionylation in human lymphocytes.

*Thioredoxin* is a protein thiol/disulfide oxidoreductase catalysing a thiol-disulfide exchange reaction. Although its most important function is to keep ribonucleotide reductase in the reduced state, and thus is essential for DNA synthesis (49), it has a broad substrate specificity and can reduce hundreds of proteins (73) with the overall reaction (1) below:

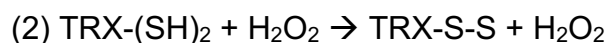


The oxidized TRX is then reduced back by the NADPH-dependent selenoprotein enzyme TRX reductase.

Under oxidative conditions, such as in the absence of TRX reductase to regenerate TRX in its reduced state, TRX can act as an oxidant (39) with the reverse of the reaction above. TRX has long been known to be secreted and was originally

described by the group of Yodoi as a leukaemia cell product that stimulates lymphocyte proliferation (89,96).

*Peroxiredoxins* are TRX peroxidases and use reduced TRX as the electron donor in eliminating  $H_2O_2$ . During its catalytic action, PRDX is first oxidized by  $H_2O_2$  and then reduced by  $TRX-(SH)_2$  with the resulting reaction (2) below:



PRDXs have a very high specificity for TRX and, in general, cannot use other proteins as substrates.

It is very interesting that both PRDXs and their substrate are co-released under inflammatory conditions. We have hypothesized elsewhere that, because the extracellular environment is an oxidizing one, lacking glutathione and NADPH, these two proteins could act in concert to oxidize other proteins (81).

Secretion of PRDXs has also been reported previously, when these proteins were originally termed natural killer cell enhancing factors (40).

*Profilin* and *vimentin* were also identified among the intracellular glutathionylated proteins in our pioneering redox proteomics study (37). Profilin has already been reported by a previous study in the human immunodeficiency virus 1 (HIV-1) - infected, monocyte-derived macrophage secretome, although that study did not investigate its redox state (54). Like profilin, vimentin was also identified previously in the macrophage secretome, although its redox state was not investigated (54,68).

#### *Other forms of thiol oxidation*

In addition to glutathionylation, disulfide-linked homodimerization has also been reported in secreted proteins. We found that PRDX1 and PRDX2 need to form disulfide-linked homodimers in order to be secreted. Mutation of the cysteine involved in homodimerization, but not of other cysteines, prevents their secretion (70).

A similar observation was made for fibroblast growth factor 1 (FGF1) where dimerization occurs during its secretion (67) and mutation of those cysteine involved in homodimerization blocks it (51).

In the case of PRDX1 and 2, disulfide-linked dimerization targets them for secretion via the exosomal route, while FGF1 is then secreted as part of a larger multimeric complex (67).

#### *Secretion of carbonylated proteins*

In terms of irreversible oxidative damage, redox proteomics studies on oxidatively damaged proteins by the Butterfield's group have found, increased carbonylation in some circulating protein in Alzheimer Disease, including haptoglobin  $\beta$  chain, serotransferrin and  $\alpha$ 2-Macroglobulin (28).

#### *The problem of cell lysis contamination in secretome studies*

Whenever studying non-classical secretion of proteins, the problem arises as to whether their presence in the cell culture supernatant is due to secretion or if it is an artefact due to leakage from a small amount of dying cells in the culture. This problem is often addressed with a shortcut, where cell toxicity is measured by Trypan blue exclusion or by assays where cell viability is assessed by a colorimetric

or fluorometric metabolic assay such as those employing the oxidation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT). The risk that release of the protein may be due to non-specific leakage from dead cells is then ruled out with statements like “cell viability was over 95%” or “cell death was less than 5%”. However, the real question is whether that 5% (or even a 0.5%) of cell death can account for the observation that a protein is present in the secretome.

We have addressed this problem by assessing not only the cell viability, as described above, but also the percentage of cell death that would be required to account for the amount of protein released in the supernatant. For instance, if the cells are cultured in 24-well plates in 1 ml of culture medium that is then analysed to measure the secreted proteins, parallel samples should be set up in the same way and the cells then lysed in 1 ml of buffer. Then comparable volumes/dilutions of the cell lysate or the supernatant should be analyzed by Western blot. Using this approach to the study of PRDX1 secretion, for instance, we calculated that at least 40% cell death would be required to account for the observed release of PRDX1 (23).

An alternative approach is to measure the release of an irrelevant intracellular protein, such as actin. Although we have done so (23), this may not work in all experimental models as in some cases actin may be also secreted (54). In general, we strongly recommend a combination of all these methods to rule out non-specific leakage.

**Methods of proteomics analysis with specific consideration to problems arising from the study of secreted proteins**

A wide range of methodologies are used to characterize cellular secretomes (69). Among them, the recent advances in liquid chromatography tandem-mass spectrometry (LC-MS/MS)-based shotgun proteomic techniques have provided powerful tools for targeted or non-targeted protein identification and quantification strategies. Conditioned media, with or without serum supplementation, has often been used in secretomics and unfortunately in such complex biological samples, the proteins of interest are generally present in small amounts, are highly diluted and may be masked by more abundant serum proteins. To overcome the problem of large dynamic range between low and high concentration proteins, the strategies of sample preparation and MS-based analysis should be adapted (see Fig. 5 for a global view of the possible strategies). Pre-fractionation of proteins (top down approach) or peptides (bottom up proteomics) with high resolution separation methods can be applied to decrease the complexity of samples. Use of the new generation of high-resolution mass spectrometry (MS) can allow quantitative in-depth large scale protein expression and characterization in complex mixtures. All the different aspects (cell culture, sample preparation, fractionation methodologies, MS technologies, quantitative profiling of dynamic secretomes, and data analysis) are discussed below.

#### *Culture media and sample preparation*

Here we discuss some general considerations of cell culture, sample concentration and cleanup that should be done with the minimum number of steps to limit the loss of protein before pre-fractionation. To avoid contamination of low-abundance proteins secreted by cells, a serum-free culture medium should be used, whenever



possible; phenol red-free media is also preferred as phenol red may interfere with MS analysis and is very difficult to eliminate. Conditioned medium should be spin at high speed (20,000 g at 4°C) in order to eliminate cell debris. Concentration of large volumes of medium can be done by freeze-drying (10) or ultrafiltration, for instance on Amicon Ultra-15 with 3 KDa cut-off membrane (Millipore); this filtration device also enables desalting, as culture media have many chemical compounds like salts and amino acids which interfere with MS through ion suppression; ultrafiltration also enables buffer exchange before fractionation or protease in-filter digestion, described as Filter Aided Sample Preparation (FASP) (100). Protein precipitation using organic solvents (such as acetone) or trichloroacetic acid is another option to achieve both protein concentration and elimination of undesirable molecules (46) although subsequent re-solubilization of the precipitated proteins may be difficult.

Prior to proteomic analysis, some authors used multiple immunoaffinity depletion methods (e.g. SIGMA ProteoPrep20) to remove some of the most abundant plasma proteins (91), or protein equalizer technology like combinatorial peptide ligand library (BioRad ProteoMiner) (77,92); sometimes the two technologies are used to allow enrichment of proteins in biological fluids for in-depth proteomic analysis (11). Independent of the strategy used, proteins need to be reduced, alkylated, digested by proteases (trypsin, chymotrypsin, LysC), labeled and desalted on C18 before MS analysis.

#### *Quantitative proteomic profiling by mass spectrometry*

Due to the complexity and broad dynamic range of proteins in cell secretomes, pre-fractionation techniques are necessary to reduce the complexity of conditioned medium and to enhance proteome coverage enabling the identification of more proteins by MS. The two main proteomic approaches, top down and bottom up, are

presented in this section. Particular attention will be given to shotgun proteomics with peptide labeling or label-free, emerging strategies that take advantage of the most recent advances in separation technology, high scan speed and high-resolution analyzers. We will also mention targeted proteomic by Multiple/Selected Reaction Monitoring (MRM/SRM).

#### *Top down proteomics*

In the offline strategy, proteins are first separated either by gel-based methods, liquid chromatography or mixed methods, digested by proteases and then analyzed by MS (nanoLCMS/MS, MALDI-TOF, MALDI-TOF/TOF).

The classical proteomic approach involves 2-dimensional gel electrophoresis (2-DE) and difference gel electrophoresis (DIGE) which enables protein separation by isoelectric focusing (IEF) according to their isoelectric points in first dimension and SDS-PAGE according to their molecular weight in the second dimension (38,106). Protein spots are then visualized by staining with Coomassie blue, silver nitrate or fluorescent dyes (such as SYPRO Ruby). After gel imaging, gels are quantitatively analyzed using an image analysis software and the spots of interest are excised from gel and treated as described above before identification by MS.

A combination of multidimensional separation methods, including C4 reverse phase liquid chromatography (RPLC) (35), off gel electrophoresis (65) and SDS-PAGE (74), can be used to separate proteins in the first dimension. Collected fractions or gel slices are then subjected to trypsin digestion. Peptides are subsequently desalted and analyzed online by nanoLC-MS/MS with an electrospray ionization interface (ESI).

Capillary electrophoresis (CE) can be directly interfaced with mass spectrometers *via* ESI and used for intact protein characterization taking advantage of an excellent

resolution size-sorted capacity and a good sensitivity (105). Top down approaches using CE allow elucidation of complex post-translational modifications to generate information on protein isoforms (59). However, top down proteomics allow limited proteome coverage identification compared with a bottom up approach.

#### *Bottom-up shotgun proteomics*

Bottom up shotgun is a modern strategy, now widely used in large scale, detailed, relative quantitative protein profiling in complex mixtures. Recent advances in MS led to the development of several methods for relative protein quantification, including isotopic labeling: stable isotope labeling by amino acids in cell culture (SILAC), isobaric tags for absolute and relative quantification (iTRAQ), isobaric tandem mass tags (TMT), isotope-coded affinity tags (ICAT) and  $^{16}\text{O}/^{15}\text{O}$ . Alternative methods include MRM/SMR, or label-free methods based on peptide intensity measurements. SILAC is based on *in vivo* incorporation of isotope labeled amino acids (Lys, Arg) into *de novo* synthesized proteins during cell culture (72). After treatment, cell culture media from the two samples are combined and subjected to sample preparation as described above. Several orthogonal separation techniques can be used: purified proteins are separated by SDS-PAGE (74) and in-gel trypsin-digested or desalted peptides are first fractionated by strong cation exchange (SCX) (87). Then, all subsequent fractions, either from SDS-PAGE or SCX, are subjected to reverse phase nanoLC-MS/MS analysis.

Other *in vitro* label-based techniques have also been used: e.g. addition of light/heavy chemicals to each protein cysteinyl residue with ICAT reagents (47), differential labeling at N-terminal group of digested peptides with iTRAQ (80), or TMT reagents (87) and labeling of digested peptides at the C-terminal with  $^{16}\text{O}/^{15}\text{O}$  (11).

Note that in these approaches, all labeled peptides are chemically identical and co-elute during the chromatographic separation step but have different masses that can be resolved by MS.

In the ICAT method, proteins from two biological samples (e.g. control cells and treated cells) are labeled independently, mixed before trypsin digestion and ICAT-labeled peptides are isolated by affinity biotin tag; enriched peptides are separated online by nanoLC-MS/MS or offline by MALDI-TOF/TOF. The relative quantification of protein amount is determined by the ratio of the peptide pairs and MS/MS fragmentation-based peptide/protein identification.

The iTRAQ and TMT methods are particularly attractive quantification techniques in shotgun proteomics, mainly due to the possibility of labeling up to eight (iTRAQ-8Plex, SCIEX) or ten (10plex TMT, Thermo Scientific) different biological samples which can then be simultaneously analyzed in a single experiment. In brief, the sample workflow is the following: purified proteins extracts are reduced, alkylated and digested, and the peptides contained in each sample are labeled by reagents, mixed together before pre-fractionation by different techniques (SXC) (93). Then, all the fractions obtained are analyzed by high-resolution nanoLC-MS/MS which identifies peptides and the relative abundance of protein in the different samples, using the ratio of reporter ions released during the fragmentation step. For either top down or bottom up proteomics, dedicated database search algorithms (Matrix Science MASCOT, Agilent Technologies SPECTRUM MILL, Thermo Finnigan SEQUEST, Bioinformatics Solutions Inc. PEAKS DB) are used for protein identification. Also for both *in vitro* and *in vivo* labeling approaches, dedicated bioinformatic tools are used for the relative protein quantification: iQuantitator (85) for

iTRAQ, MaxQuant (97) and MassChroQ (98) for SILAC are cited as examples (see Fig. 5 for more details).

Besides labeling-based techniques, label-free quantification has become the new generation in shotgun proteomic strategies, taking advantage of recent advances in separation technology and in MS. At present, identification of 5,000 proteins is attainable in a standard 90-min gradient with nanoLC ultra-high-pressure HPLC system coupled online to modern high resolution mass spectrometers (5,82). Two MS label-free data analysis pipelines can be applied. The first uses the spectral count, which is based on the frequency number of identified total MS/MS spectra of the same protein in each of the datasets (27). The second approach is based on measurement of changes in chromatographic peptide ion intensity, detecting peptides by MS, matching the corresponding peptides across multiple LC/MS runs using their mass-to-charge and retention time, selecting discriminatory peptides by matching peak area integration on extracted ion chromatograms with specific software: Progenesis Q1 (Nonlinear Dynamics), Mass Profiler Professional (Agilent Technologies), MaxQuant (Max Planck Institute of Biochemistry) and MassChroQ. The final step is identification of regulated peptides by targeted MS/MS. Label-free MS strategies have many advantages, including low cost, high-throughput (no limit to the number of samples and conditions that can be compared), time, as sample preparation is easy (based on SDS-PAGE stacking gel purification/in-gel digestion (99) or in-filter digestion using FASP (101)).

### *Targeted proteomics*

As part of a discovery strategy, SRM, also called MRM, is a method of absolute quantification in targeted proteomic analyses and is usually carried out on triple-quadrupole mass spectrometers (QQQ). In this method, isotopic absolute

quantification (AQUA), peptides are spiked in complex samples and used as internal standards (41,95). AQUA peptide is designed to be identical to the prototypic target peptide generated by sample digestion. Both peptides co-elute in a known retention time window and are analyzed simultaneously. The concentration of the target peptide is then determined by measuring the signal response for the target peptide relative to that of the AQUA peptide. In the MRM mode, a particular pair of parent ion and several fragments are selected and detected to ensure the specific detection of low-abundance proteins in highly complex mixtures. The SRM method is specific, highly sensitive and is a useful tool for high-throughput screening of biomarkers in a very large number of clinical samples.

### **Redox arrays**

We soon realized, in the course of our studies on redox proteomics of glutathionylated proteins, that some proteins are found over and over in proteomics studies. In fact, this is a very well-known bias of the proteomics approach. While MS is very sensitive, in a mixture of proteins the abundant ones are identified more easily and can mask the less abundant ones (75,78). For instance, PRDXs account for up to 1% of total cellular proteins (102). It is very likely that we would miss most of the cytokines or components that are present in the secretome at concentrations in the range of picogram/ml.

We have tried to circumvent this problem setting up a technology that we called “redox arrays” and that are based on commercially-available antibody arrays (71). These are membranes or glass slides coated with antibodies to up to 1,000 different proteins and are used to identify, in a semiquantitative manner, proteins that are

differently expressed in different samples. We have adapted this approach to identify proteins undergoing glutathionylation in the inflammatory secretome. This strategy is outlined in Fig. 6. The first part is based on the use of BioGEE to attach a biotin tag to glutathionylated proteins described above (section 2.3). Then the supernatant, containing biotinylated (glutathionylated) and non-biotinylated (non glutathionylated) proteins, is applied to a membrane array (we used an antibody arrays consisting of 1,000 antibodies immobilized on nitrocellulose membrane by Raybiotech, Norcross, USA), the membranes are washed as per the manufacturer's instruction but taking care of avoiding the use of any reducing agent (included in most protocols) that would remove the biotin tag. Detection with streptavidin-peroxidase followed by enhanced chemiluminescence (ECL) will reveal a dark spot if a protein is released as glutathionylated. DTT-treated samples will allow identifying false positive spots. If a protein is released but not glutathionylated, it will not show up.

Using this approach, both for the secretome and for the cell lysate, we could identify 80 potential glutathionylated proteins, most previously unrecognized to be glutathionylated. We use the word "potential" because the same caution should be used as in the MS identification and require validation as described above (section 2.2). In our methodological study we validated two such proteins, soluble IL-1 receptor type II (IL-1sRII) in the secretome and the tyrosine-protein kinase Lyn in the intracellular fraction (71).

We have tried to establish if this method would allow the identification of low abundance proteins and compared the proteins identified with this study (71) with our previous study using redox proteomics based on 2D gels (37), that of the study mentioned in section 2.3.1 based on the labelling with BioGEE followed by affinity purification on streptavidin and shotgun proteomics (23), as well as with a curated

database, dbGSH, of known glutathionylated proteins identified so far (<http://csb.cse.yzu.edu.tw/dbGSH/>) (24). We then used a published list of over 7,000 human proteins ranked by their abundance in a human osteosarcoma cell line (4) to calculate the expected frequency distribution of human proteins according to their abundance. Although these data are only indicative, as they are obtained in different cells, ranging from human T lymphocytes (37) to mouse macrophage (23,81) and human monocytic leukemia cells (71), still the study on the osteosarcoma cell line we refer to has PRDX1, vimentin and profilin in the 20 most abundant proteins (4). The frequency distribution in the reference study is shown in Fig.7A. We refer to this as the “expected” frequency distribution of proteins with different abundance and compare with that found in the various redox proteomics experiment of glutathionylated proteins performed by us from 2002 to date, as well as that of the proteins present in the curated database. This was done on the assumption that the protein abundance is similar across the different cells, which is of course an approximation.

Fig.7B compares the distribution of glutathionylated proteins found with our first method based on radiolabeling of GSH with  $^{35}\text{S}$ , 2D electrophoresis followed by autoradiography, spot picking following Coomassie blue staining and MS identification (37). It can be seen that the proteins identified are heavily skewed toward the high-abundance ones, as one may expect from a study using Coomassie blue staining. The identification appears less skewed in our more recent approaches based on the use of BioGEE and MS identification (23,81) (Fig.7C). It is improved further in the curated database that clearly includes mainly proteins from proteomic studies but also proteins from studies aimed at specific proteins, and not involving their identification by proteomics (Fig.7D). The frequency distribution obtained with



the redox array method (taken from (71)) is surprisingly close to the expected distribution for an unbiased method (Fig.7E). Needless to say, this approach is far from being unbiased as the proteins for which the antibodies are spotted on the arrays are selected based on some criteria. For instance, if we were to use an antibody array for cytokines and growth factors, the frequency distribution would be skewed in favour of low-abundance proteins because most cytokines are not expressed constitutively and, even when induced, are in the picogram range.

We think that the strategy based on redox arrays can be tailored to identify other forms of oxidation or transient disulfides. For instance, it could be coupled to biotin switch approaches using reductants specific for S-nitrosylated proteins (52). We believe this technique might represent, for the field of redox biology, an equivalent to the arrays used to study the kinome and the phosphoproteome.

## **Biological significance**

### *Biomarkers of the redox state*

The most obvious relevance of the redox secretome is in biomarker discovery. In fact, secreted proteins are likely to be detectable in circulation – an almost essential feature for a successful biomarker. Because reactive oxygen species are short-lived, it is impossible to measure any of them in biological fluids and we have to rely on biomarkers as proxy indicators. Oxidized proteins (such as carbonylated proteins or glutathionylated proteins) can be an indicator of the redox state of the organism provided they are prepared properly. In particular, for thiol-oxidized proteins it is essential to treat the sample with NEM or another alkylating agent before storage, in

order to prevent thiol-disulfide exchange reaction with protein thiols or small-molecular weight thiols.

Although not a secreted protein, many studies have measured glutathionylated haemoglobin in many disease conditions and found an association with some of them (reviewed in (45)). The same approach could be used for secreted proteins. Oxidized proteins could be of the same use as glycated haemoglobin in diabetes, that does not just reflect to the level of glucose at a given time point but really is an indicator of the exposure of the organism to high glucose (21). As mentioned earlier, the example of PTX3 suggests that the redox state of a biomarker may represent an additional dimension, improving its diagnostic or prognostic significance (29).

### *Functional significance*

We think the study of the redox secretome can lead to the identification of novel soluble mediators (cytokines). Probably the best example of this is thioredoxin which was discovered by Arne Holmgren in Stockholm as a reducing cofactor essential for the catalytic action of ribonucleotide reductase and by Junyi Yodoi in Kyoto as an adult T-cell leukaemia derived factor; Yodoi was expecting to have identified an interleukin-1 gamma but once the protein was sequenced it was identified as TRX (104). Also PRDXs were first identified as natural killer (NK) cell activators even before their enzyme activity was established (85). The main cytokine-like activities of redoxins are summarized in Table 2.

The identification of inflammatory cytokines has been a great success story: tumor necrosis factor (TNF) was discovered as an inflammatory mediator in 1985 (7) and

by 1998 anti-TNF antibodies were approved for use in inflammatory disease. Other cytokines inhibitors followed to block interleukins (IL-) IL-6, IL-1 and IL-17.

It may be worth noting that most known cytokines have been discovered either by *in-silico* mining or by biochemical purification of proteins with a biological activity. So far, we think that the only “cytokine” discovered by proteomics is HMGB1, which was observed as it was identified in Coomassie blue stained gels as differentially expressed in the supernatant of macrophages stimulated with LPS compared to unstimulated ones (97). Thus, the study of the redox secretome may lead to the identification of previously unrecognized mediators and potential pharmacological targets.

As mentioned above, PRDX1, PRDX2 and FGF1 are secreted via disulfide-linked homodimerization. It will be interesting to investigate whether other proteins are secreted via formation of a disulfide-linked dimer as this may eventually describe a family of proteins whose secretion is dependent on the redox state of the cell and/or the extracellular environment.

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## LIST OF ABBREVIATIONS

2-DE=two-dimensional electrophoresis

AQA= absolute quantification

BioGEE= biotinoylated GSH ethyl ester

CE= Capillary electrophoresis

Cys=cysteine

DIGE=difference gel electrophoresis

DTT= dithiothreitol

ECL= enhanced chemiluminescence

ELISA, enzyme-linked immunosorbent assay

ESI= electrospray ionization interface

FASP= Filter Aided Sample Preparation

FGF1= fibroblast growth factor 1

GSH=glutathione (reduced)

GSSG=glutathione disulphide

HMGB1= High-mobility group protein box 1

HPLC=high-pressure liquid chromatography

ICAT= isotope-coded affinity tags

IEF= isoelectric focusing

IL=interleukin

IL-1sRII=soluble IL-1 receptor type II

ITRAQ= isobaric tags for absolute and relative quantification

LC-MS/MS=liquid chromatography tandem-mass spectrometry

LPS=lipopolysaccharide

MALDI= matrix-assisted laser desorption/ionization

MRM = multiple reaction monitoring

MS=mass spectrometry

MTT=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

NEM, N-ethyl-maleimide

PRDX, peroxiredoxin

PTX3=pentraxin 3

QQQ= triple-quadrupole

RPLC= reverse phase liquid chromatography

SCX= strong cation exchange

SDS-PAGE= sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SILAC= stable isotope labeling by amino acids in cell culture

SRM= selected reaction monitoring

TMT= tandem mass tags

TNF=tumor necrosis factor

TOF=time of flight

TXN=thioredoxin

## Tables

Table 1. Glutathionylated proteins in the inflammatory secretome

<b>Protein</b>	<b>No of cysteines</b>	<b>Notes</b>
Thioredoxin-1	5	
Peroxiredoxin-1	4	Dimer
Peroxiredoxin-2	3	Dimer
Profilin-1	3	
Vimentin	1	

Table 2. Cytokine-like activities of redox enzymes

Enzyme	Secretion	Circulating	Extracellular Activity
Thioredoxin TRX80 (truncated thioredoxin)	Leukaemia cells Macrophages, tumour cells	Several diseases	T cell proliferation and activation (1,96) Chemotactic activity (6)  Eosinophil cytotoxicity-enhancing factor (2)
Glutaredoxin	Monocytes	Plasma (64); diabetes	Cytoprotection NF-kB activation (in neurons) (31)
Peroxiredoxin 1/2	Monocytes	Various diseases	Proinflammatory (81,86) NK cell enhancing factors (85)
Protein disulfide isomerase		Various cells (48)	Integrin regulation, thrombus formation (25)
Glutathione peroxidase 3	Muscle cells (26) (leader peptide)		H <sub>2</sub> O <sub>2</sub> detoxification (8)



## FIGURE LEGENDS

**FIG. 1. Different redox states of protein cysteines.**

**FIG. 2. Glycolytic enzymes targeted by glutathionylation.** The figure shows the enzymes undergoing glutathionylation and those whose enzymatic activity, according to previous literature, is affected by thiol oxidation. Based on ref. (44).

**FIG. 3. Concentration of different thiols in cells and plasma.**

cysteine free cysteine; GSH, glutathione (reduced); PSSC, cysteinylated proteins; PSSG, glutathionylated proteins.

**FIG. 4. Affinity-enrichment of glutathionylated proteins for proteomics studies.**

Elution of proteins with DTT reduces the disulfide bond thus eluting only those proteins attached through glutathionylation.

**FIG.5. Workflow of MS-based proteomic provided tools for targeted or non targeted protein identification and quantification approach.** Different techniques used: e.g. samples preparation, proteins/peptides pre-fractionation methods, MS-based proteomic approaches and data analysis tools are presented.

**FIG.6. Workflow for redox arrays that identify glutathionylated proteins.**

**FIG. 7. Different recovery of low-abundance glutathionylated proteins with different techniques.** Panel A shows the expected frequency distribution; panels B-E show the observed frequency distribution from the studies referred to in the text compared to the expected one. References are: expected frequency distribution, Beck et al 2011 (4); 2D-gel approach, Fratelli et al., 2002 (38); BioGEE/MS approach, Checconi et al. 2015 (23); dbGSH, (<http://csb.cse.yzu.edu.tw/dbGSH/>)(24); redox arrays, Mullen et al. 2015 (70).

PSH	<b>-2</b>	Free thiol
PS-SP PS-SG PS-SC	<b>-1</b>	Disulfide bond (intra- or inter-chain) Mixed disulfide (glutathionylation) Mixed disulfide (cysteinylation)
PS-NO PSOH	<b>0</b>	S-nitrosothiol Sulfenic acid
PSO <sub>2</sub> H	<b>+2</b>	Sulfinic acid
PSO <sub>3</sub> H	<b>+4</b>	Sulfonic acid (irreversible oxidation)

Fig.1

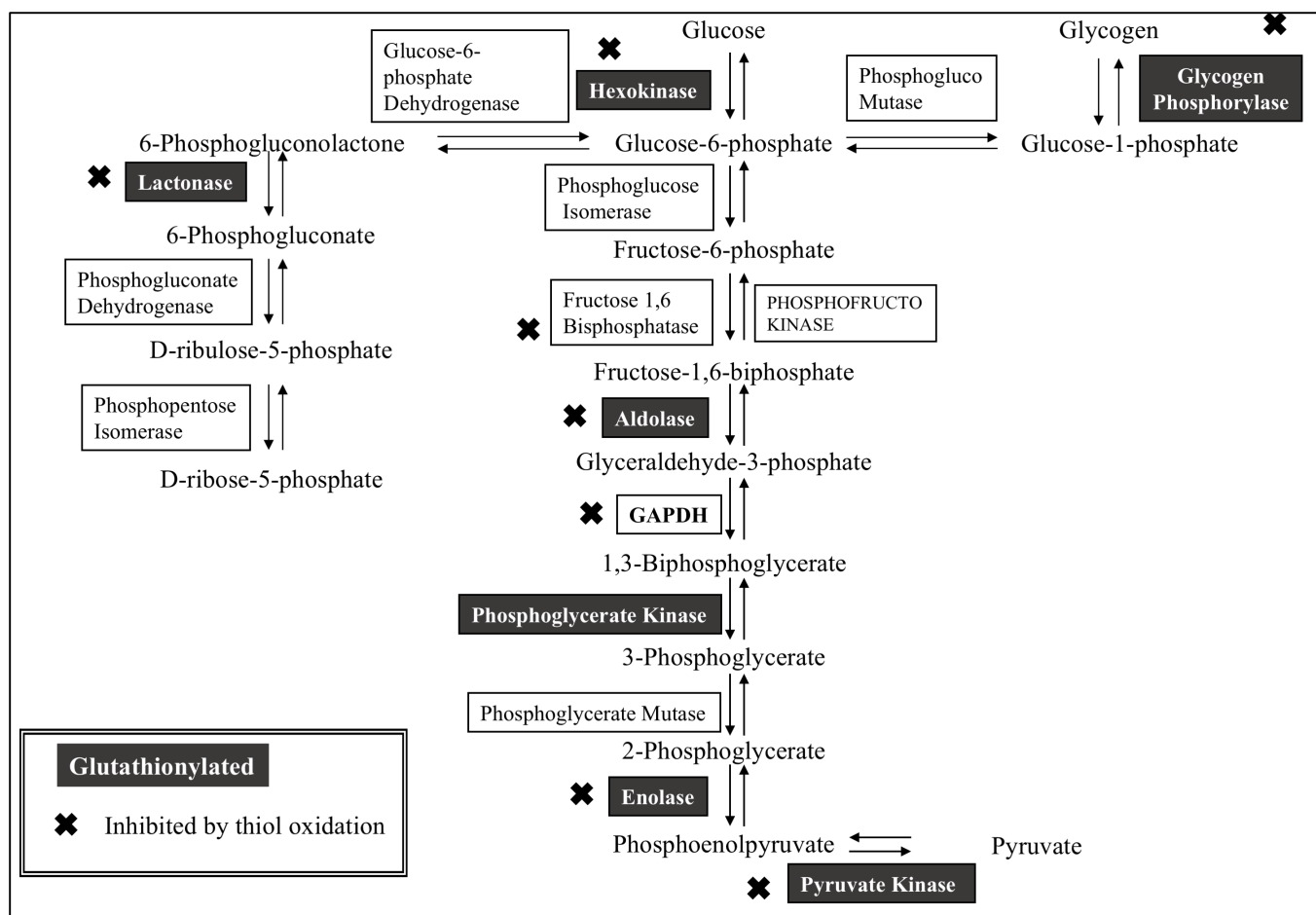


Fig.2

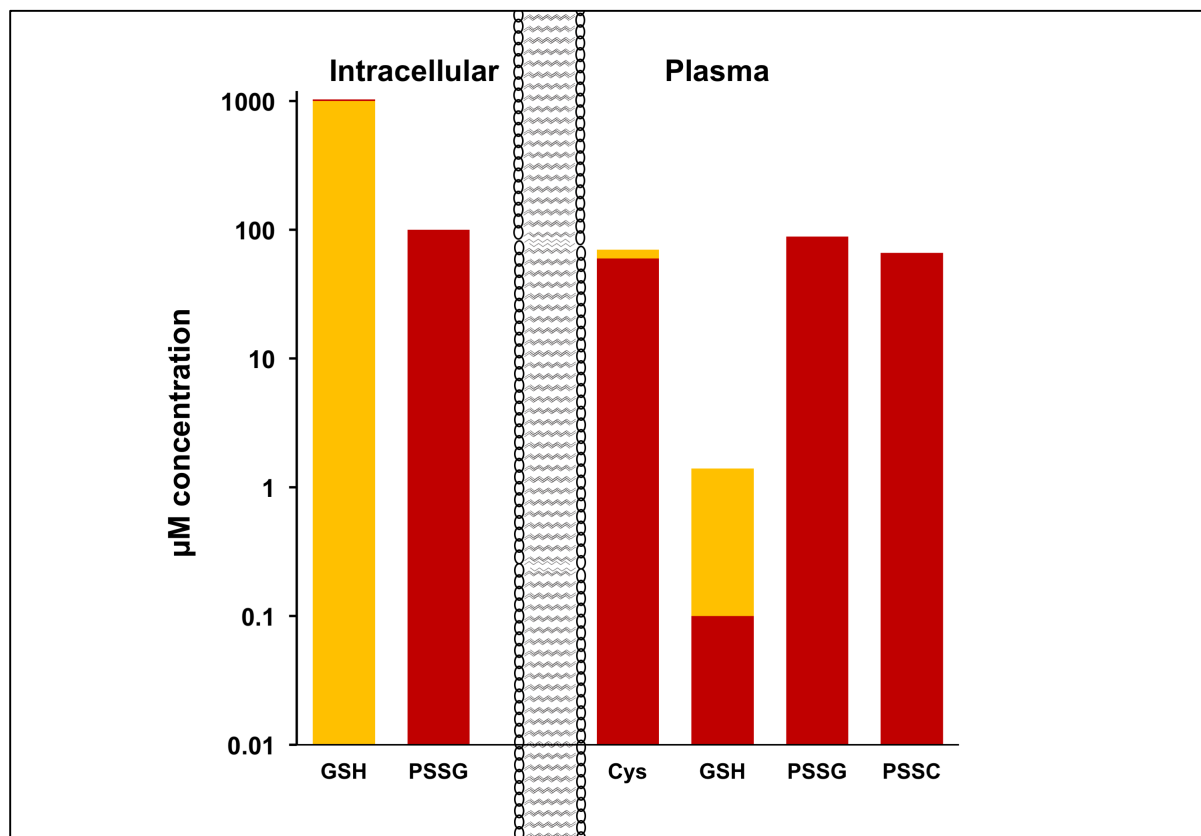


Fig.3

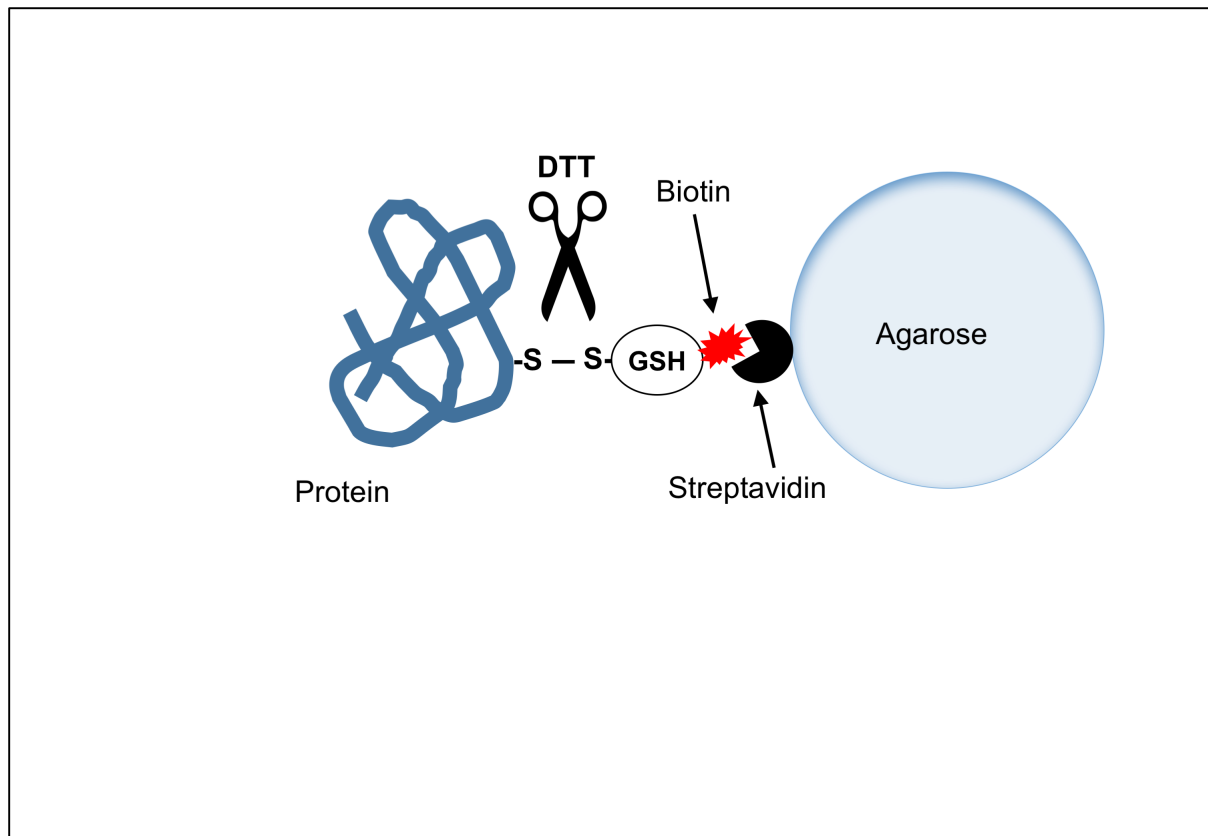


Fig.4

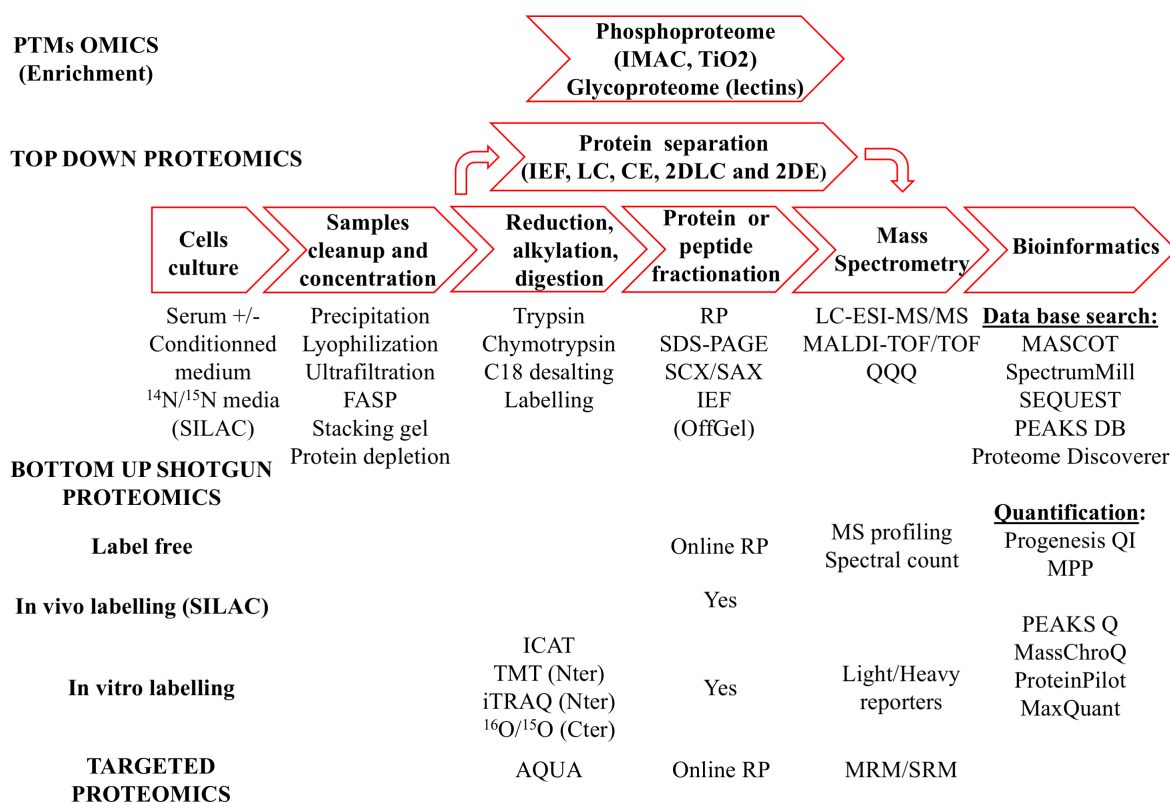


Fig.5

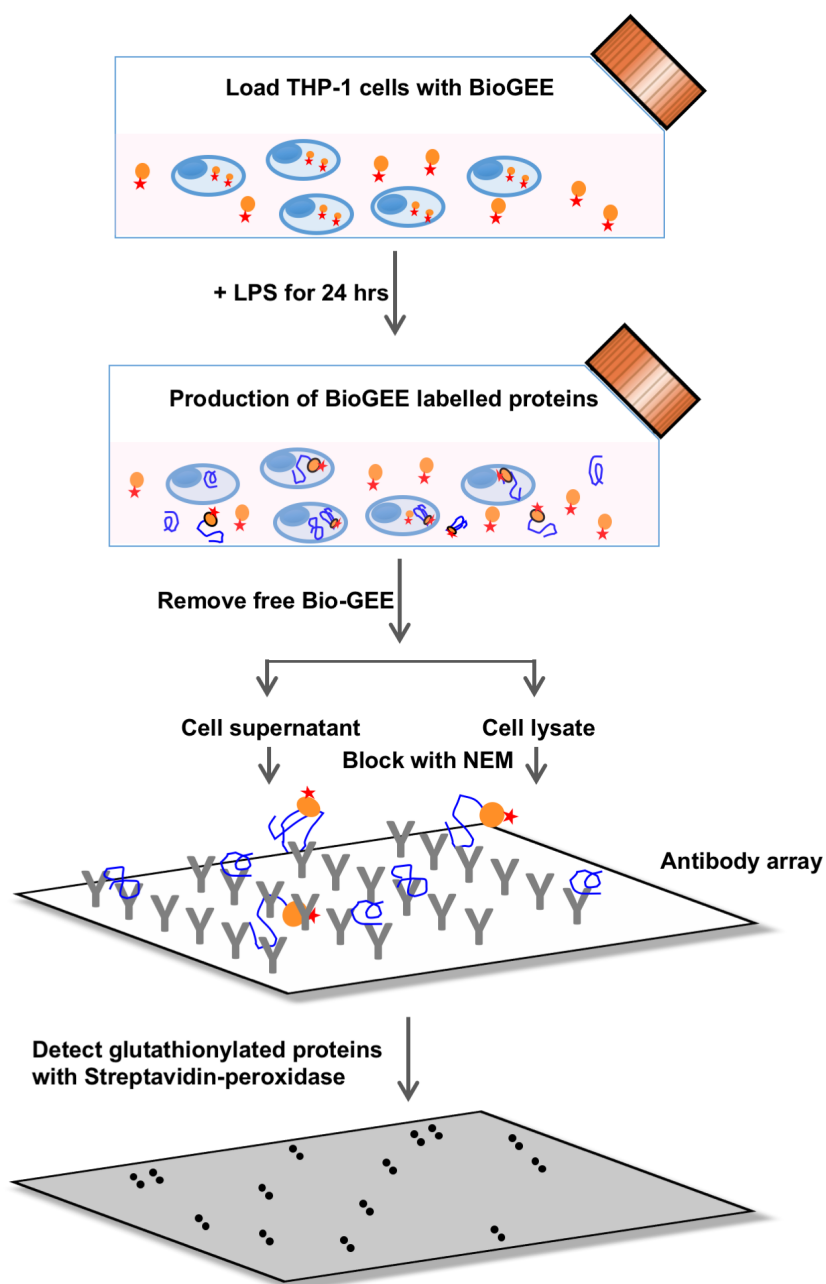


Fig.6

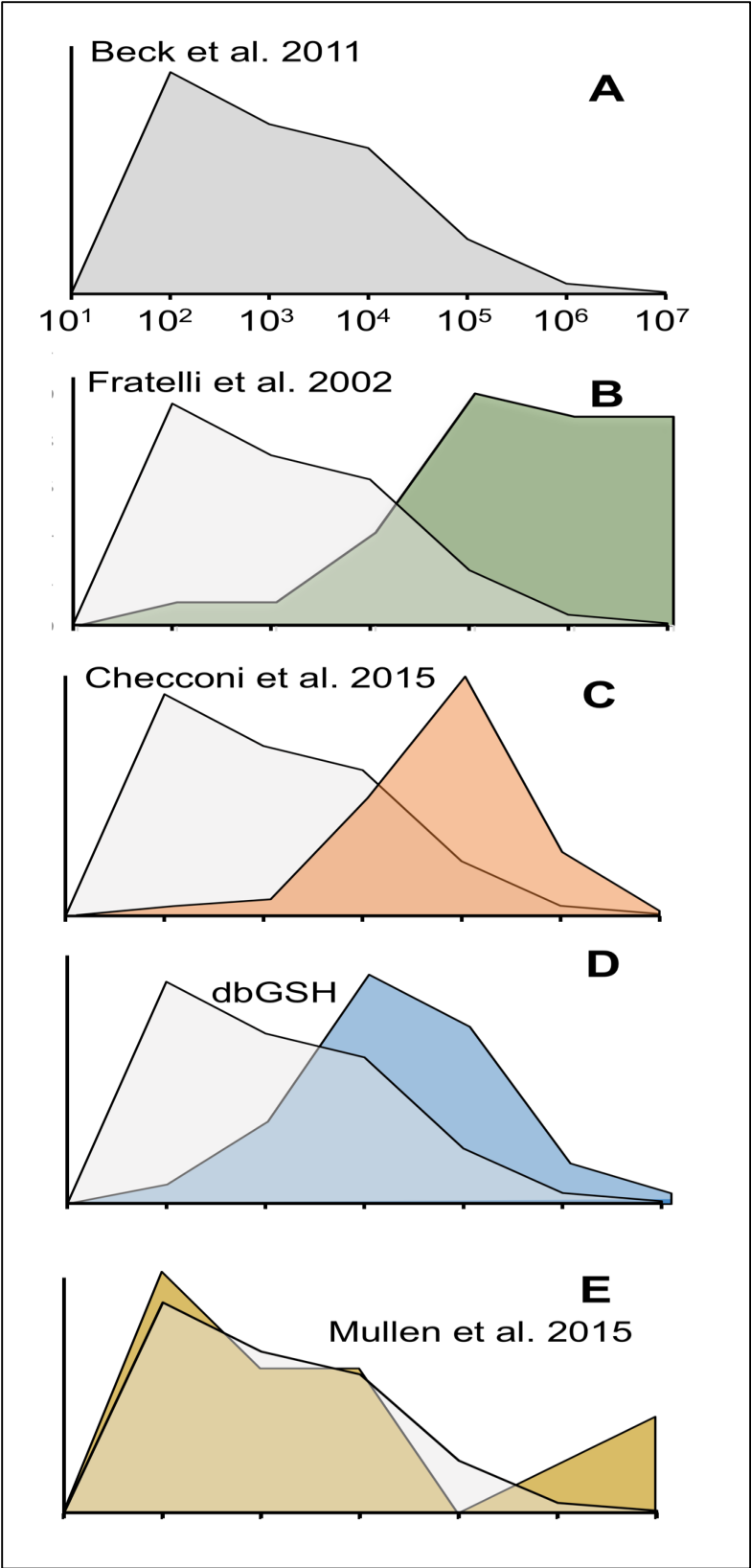


Fig. 7



